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(54) **WEIGHTED MICROSPONGE FOR IMMOBILIZING BIOACTIVE MATERIAL.**

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Description

This invention was made in the course of, or under, a contract with NIH. The government has rights to the invention pursuant to SBIR Grant No. CA37430.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention pertains to the art of immobilizing bioactive materials and particularly relates to an improved microsponge for use in motive bioreactor systems. The present invention also relates to the art of culturing microorganisms and cells, hereinafter referred to collectively as organisms, and particularly relates to the culturing of organisms immobilized on and/or in microsponges in motive reactor systems as submerged suspensions.

Description of the Prior Art

A large variety of chemical products are produced by culturing organisms. For example, fermentation processes abound for producing antibiotics and other drugs, alcoholic beverages, cheeses and the like. Most fermentation processes, however, are carried out commercially using batch reaction procedures, even though the value of continuous culturing techniques are recognized.

Recent advances in the areas of genetic engineering and medicine have ushered in a new era in the production of chemical products by culturing organisms. For example, hybridized mammalian cells or hybridomas have been developed which secrete antibodies specific to known antigens, such as tetanus toxoid. Bacteria, such as E. coli, have been genetically engineered to produce specific proteins, such as insulin. To best utilize this new technology, however, techniques superior to the batch fermentation processes must be developed for continuously culturing such organisms at high concentration and under conditions of optimal growth so as to maximize the production of such products.

Various arrangements for immobilizing bioactive materials are known. Solid supports have long been used for immobilizing microorganisms in the treatment of waste water and related fermentation processes. More recently, solid microcarriers have been used to obtain high cell densities in the culture of attachment-dependent cells. For example, microporous polymeric supports fabricated for example from dextran have been used for cultivating cells. Such supports can be obtained commercially from Pharmacia Fine Chemicals under the brand name Cytodex®. Such solid bio-supports, however, are not suitable for motive reactor systems such as vigorously stirred tanks and fluidized beds since substantially all of the cells are adherent to the surface of said supports and thus are exposed to impact stress and trauma during operation.

Porous inorganic microcarriers also are known and such supports potentially provide protection for the cells in motive applications since the cells populate the interior of the microcarriers. Unfortunately, inorganic microcarriers cannot be made with the proper combination of permeability and specific gravity to function well in all motive applications. For example, the porous fritted glass or cordierite supports described in Messing et al. U.S. 4,153,510 would typically exhibit specific gravities in aqueous suspension of less than about 1.3 if their void fractions are greater than about 80% (Note that void fractions for the Messing supports are not disclosed). Quite understandably, these supports are not suitable for all motive reactor systems where a higher specific gravity generally is needed to ensure high relative velocities for maximum rates of mass and energy transfer. Consequently, these supports have generally been relegated for use in packed bed applications.

Additionally, it is not known how suitable these prior art microcarriers are for the cultivation of attachment-independent organisms such as hybridomas.

An object of the present invention is to provide a microsponge containing immobilized bioactive materials suitable for use in motive reactor systems.

Another object of the present invention is to provide a microsponge suitable for immobilizing a large variety of organisms characterized by wide variations in size and their degree of attachment to solid supports.

A further object of the present invention is to provide a microsponge suitable for motive reactor systems which permits the continued growth and reproduction of immobilized organisms.

It also is an object of the present invention to provide a microsponge suitable for motive reactor systems which is conducive to maximizing the metabolic activity of immobilized organisms.

Yet another object of the present invention is to provide a method for continuously culturing organisms at high concentrations.

Still another object of the present invention is to provide a microsponge suitable for motive reactor systems which permits the culturing of organisms at high concentrations while accommodating either maximum growth rate or maximum metabolic activity.

Another object of the present invention is to provide a method for the in vitro culturing of both attachment-dependent and attachment-independent organisms.

These and other objects of this invention will become apparent from a consideration of the specification and appended claims.

SUMMARY OF THE INVENTION

The present invention pertains to a weighted microsponge, (preferably a collagen microsponge) for immobilizing bioactive materials in motive bioreactor systems, said microsponge comprising a porous, biostable, highly crosslinked collagen matrix containing an inert weighting material, said collagen matrix having an open to the surface pore structure with an average pore size in the range of from about 1 micron to about 150 microns, with the pores of said matrix occupying from about 70 to about 98% by volume of the microsponge, said microsponge also having an average particle size of from about 100 microns to about 1000 microns and a specific gravity of above about 1.05

The present invention also relates to a method for the continuous in vitro culture of organisms for the production of biochemical products comprising the steps of:

(a) providing a highly crosslinked, collagen microsponge prepared by

(i) milling a source of collagen selected from the group consisting of Types I, II and III collagen,

(ii) admixing said milled collagen with an acidic liquid medium,

(iii) lyophilizing said collagen-liquid medium mixture into a dry sponge matrix, and

(iv) crosslinking the collagen in said sponge matrix by a treatment selected from the group consisting of:

(1) contacting said collagen with a crosslinking agent selected from the group consisting of carbodiimides and bifunctional succinimidyl active esters,

(2) subjecting said dry sponge matrix to elevated temperatures under a vacuum, and

(3) a combination thereof;

(b) inoculating the highly crosslinked, collagen microsponge of step (a) with a culture of said organisms;

(c) passing a nutrient medium in direct contact with the inoculated, highly crosslinked, collagen microsponge; and

(d) recovering said biochemical products with the nutrient medium effluent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photomicrograph showing a suitable collagen microsponge matrix of the present invention illustrating a fibrous structure.

Figure 2 is a photomicrograph of another collagen microsponge matrix according to the present invention illustrating a leafy structure.

DETAILED DESCRIPTION

The present invention is directed to a weighted microsponge, preferably a collagen microsponge, containing immobilized bioactive materials, particularly organisms, suitable for use in motive bioreactor systems and to a method for the continuous in vitro culture of organisms for the production of biochemical products. As used throughout the specification and claims, the term "bioactive material" broadly encompasses both enzymes and other chemical factors such as chelating agents, hormones, antibodies, etc., and organisms, i.e., microorganisms and the cells of higher organisms. As used throughout the specification and claims, the term "organism" broadly encompasses both mircoorganisms and the cells of higher organisms. The organisms may be derived without limitation from such diverse sources as bacteria, fungi, viruses, algae, yeasts, animal cells (tissue), e.g., mammals, insects and fish and plant cells. The term "organisms" and "cell" will be used interchangeably throughout the specification and claims. As also used throughout the specification and claims, the term "biochemical product" refers not only to both primary and secondary metabolites produced by such organisms, but also to the cellular material or biomass of the organism itself, containing, for example, non-secretory products. Since the weighted microsponge of the present invention has particular advantages when used for culturing organisms, it generally will be described with reference to such embodiments, although it is not to be so-limited.

The microsponge of this invention is prepared from a biocompatible (e.g., non-toxic) polymer that is stable in service for an appropriate period of time, e.g., on the order of months. The preferred microsponge of this invention is formed of a highly crosslinked collagen. Biocompatibility refers to the ability of the polymeric (e.g. collagen matrix material to support a viable culture of organisms without substantially adversely affecting any desired characteristic of the immobilized organisms, e.g., in the case of hybridomas, the polymeric (e.g. collagen matrix material must not reduce the production of monoclonal antibodies. The stability or biostability of the matrix material refers to its ability to maintain its strength and integrity under in vitro

also horse tendon

conditions over the relevant time period for culturing the organism of interest. For example, in the case of a hybridoma culture for producing monoclonal antibodies, it is expected that the motive bioreactor would be operated continuously for three to six months or more. Thus, the matrix material should be biostable for this time period.

Both natural and synthetic polymeric materials can be used as the matrix material. Examples of suitable polymers include polysaccharides such as dextran, dextrin, starch, cellulose, agarose, carrageenan and the like; proteins such as collagen and the like; and synthetic polymers such as polyvinyl alcohols, polyacrylates, polymethacrylates, polyacrylamides, polyesters, polyurethanes, polyamides and the like. Generally, a material's biocompatability and biostability can be verified using routine experimentation.

Based on its biocompatibility and strength, collagen is presently the material of choice. Collagen is a biodegradable polymer found in animals, including man. It has numerous uses in the medical art and in most applications is reconstituted and crosslinked into an insoluble form using various crosslinking agents, such as aldehydes, e.g., glutaraldehyde and formaldehyde; ethylchloroformate; dimethyl adipimidate; N,N-methylenebisacrylamide; 1,2-diacrylamide ethyleneglycol; cyanamide; N-N'-diallyltartardiamide; cyanogen bromide; concanavalin A; 6-aminohexanoic acid; 1,6-diaminohexane; succinimidyl active esters; carbodiimides and compounds having similar crosslinking groups and/or physical treatment techniques such as freeze-drying and severe dehydration at a vacuum of about 50 millitors or more and at a temperature ranging from 50 °C to 200 °C. Unfortunately, many if not all of the commonly used crosslinking agents, particularly glutaraldehyde, which are unavoidably present in the crosslinked collagen, cause adverse biological effects and so are cytotoxic.

Recently, a new collagen-based matrix of improved biostability has been discovered. This collagen material is prepared without the conventional crosslinking agents. In this matrix, Type I, II or III collagen is crosslinked using a carbodiimide or a succinimidyl active ester and/or severe dehydration conditions at temperatures ranging from 50 ° to 200 °C. Such crosslinked collagen typically has a molecular weight of from about 1×10^6 to in excess of 50×10^6. The molecular weight of the collagen between adjoining crosslinks varies from about 1000 to 100,000 via the formation of covalent bonds. Because of its resistance to degradation by collagenase and other proteinases, this crosslinked collagen has been found to be particularly suitable as the porous matrix of the microsponge. In fact, when used for the continuous culture of immobilized organisms, particularly hybridoma cells expressing monoclonal antibodies, microsponges made from this collagen material have exhibited some surprising properties. For example, hybridomas cultured with essentially protein-free medium are much more effective in producing monoclonal antibodies when immobilized on and/or in such collagen matrix microsponges than when such matrix is not present. Furthermore, microsponges made from the noted crosslinked collagen material appear to preferentially retain high concentrations of living (viable) cells and expel nonviable cells.

The preferred crosslinked collagen can be prepared from both soluble collagens and insoluble collagens of the Types I, II and III. The soluble collagens are prepared by limited enzymatic digestion and/or extraction of tissue enriched in such collagen types. Insoluble collagens are derived from the following typical sources: Type I collagen: bovine, porcine, chicken and fish skin, bovine and chicken tendon and bovine and chicken bones including fetal tissues; Type II collagen: bovine articular cartilage, nasal septum, sternal cartilage; and Type III collagen: bovine and human aorta and skin. For example, Type I collagen from bovine corium may be used. It is preferred to use Type I tendon collagen.

In the broad practice of the present invention, the physical form or macro-configuration of the crosslinked collagen microsponge is not critical. The microsponge can be used in various physical shapes including beads, flakes, discs, fibers, films, coatings, e.g., on extended surface supports, and sheets. For example, the microsponge can be provided as a coating on such well-known supports as pellets, stars, spiral rings, cross partition rings, Raschig rings, Berl saddles, Intalox saddles, pall rings, Tellerette rings and the like. The microsponge also can be provided in sheet form, in tubular form or in any other unsupported structure.

In the broad practice of the process of the present invention, the size of the microsponge is not critical. For example, for packed bed or fixed bed applications (e.g., plug flow reactors) the microsponge may be provided in a size typical for such applications. In order to be suitable for culturing high concentrations of organisms in motive reactor systems and allow for the transfer of nutrients to the immobilized organisms and the transfer of desired products from the microsponge, the weighted microsponge, preferably collagen, of the present invention must satisfy several functional requirements. The microsponge typically is in the shape of a bead and should have a particle size within the range of about 100 microns to 1000 microns, preferably from about 200 microns to 500 microns. At larger particle sizes the entire internal

volume of the porous structure is not utilized effectively for producing the desired product by reaction between the immobilized bioactive material and the liquid medium contacted therewith, thus degrading the volumetric productivity of the motive reactor employing such microsponges. Smaller particle sizes present practical problems in preparing the microsponge and in operating the motive reactor.

Permeability of the microsponge is another important consideration. A microsponge's permeability is determined by the interrelationship of its porosity or void fraction and its pore structure. Void fraction is defined as the ratio of the volume of interstices of a material to the total volume occupied by the material and often is expressed as a percentage. In order to permit operation at high organism concentrations, the microsponge should have a void fraction of between about 70% and 98%. Preferably the void fraction of the collagen microsponge is greater than 85% and most desirably is greater than about 90%.

The microsponge also must possess an open to the surface pore structure. This allows for cell entry, without excessive shear forces, cell retention, subsequent cell growth, and expulsion of excess cell mass. For example in cases where the desired product is not secreted by the organisms, e.g., genetically engineered E. coli with a non-expressed rDNA product such as insulin, the organisms must be able to escape the microsponge as the immobilized colony expands by division. An open pore structure is essential if this process is to proceed on a continuous basis, without rupturing the microsponge structure. The desired organism product is recovered as an entrained component of the culture harvest liquor.

The microsponge should contain pores with an average size within the range of about 1 micron for the smallest microbes and for viruses, up to about 150 microns for large mammalian and plant cells. Generally, the pores of the microsponge must be at least as large as the smallest major dimension of the immobilized bioactive material but less than about 5 times the largest major dimension. Preferably, the pore size of the matrix is on the order of 1.5 to 3 times the average diameter of the organism or cell. If unknown, the smallest and largest major dimensions of an organism can be determined using known techniques. Applicants have found that the recited combination of particle sizes and pore sizes insure adequate mass transfer of constituents such as nutrients to the immobilized organisms, as well as adequate mass transfer of constituents, such as desired metabolites from the immobilized organisms.

For use in motive reactor systems, the microsponge (e.g. collagen) also must be weighted. The polymeric materials (e.g. crosslinked collagen) used as the matrix material in the present invention generally have a specific gravity of about 1.0 or less. For proper operation in a motive reactor, a specific gravity of above about 1.05, preferably above about 1.3 and most preferably between about 1.6 and 2.0 is desired. It has been found surprisingly that it is possible to obtain collagen microsponges of the proper specific gravity by introducing certain weighting additives into the microsponge without undesirably reducing its void fraction. The weighting additive must be substantially inert in the reactor environment and non-toxic to the immobilized organism, or must be suitably treated to render the additive non-toxic. Also, the weighting additive should not adversely affect the productivity of the immobilized organism. Generally, materials, such as metals and their alloys and oxides, and ceramics, having a specific gravity above about 4 and preferably above about 7 are used. Examples of suitable weighting additives for use in the broad practice of this invention are chromium, tungsten, molybdenum, cobalt, nickel, titanium and their alloys, e.g., Monel, 316 stainless, Vitalium (a cobalt alloy with chromium and molybdenum), titanium 6Al-4V (a titanium alloy with aluminum and vanadium) and Haynes Stellite Alloy 25 (a cobalt alloy with chromium nickel, tungsten and manganese). Many of these materials, however, may not be compatible with certain organisms and routine experimentation will be necessary to assess toxicity for any application. For example, titanium is the weighting material of choice with hybridomas, since most other metals are cytotoxic.

The weighting additive can be introduced into and dispersed throughout the microsponge as a finely divided powder, with most particles having a size on the order of 10 to 40 microns. However, to minimize the surface area of the weighting additive, it is desirable to employ it as a solid core in the microsponge. Sufficient weighting material is added to yield a microsponge with the desired specific gravity. For example, about a 50 micron diameter core of a weighting additive having a specific gravity of about 7.0 coated with a 50 micron thick layer of collagen having an average pore size of 20 to 40 microns and a void fraction of about 99% yields a microsponge with a specific gravity of about 1.7 having an overall void fraction of about 85%. Such a microsponge is particularly suitable for use in an aerobic motive reactor systems.

Finally, for motive applications the collagen microsponge should exhibit the proper resistance to attrition. A charge of microsponges preferably should have a useful life on the order of three to six months or more. Typically, the microsponges should exhibit not greater than about a 10% loss in volume after three months of operation.

#4

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 5466**
Alfred SCHAUFLER	:	Docket No. 2002-0094A
Serial No. 10/054,889	:	Group Art Unit 1762
Filed January 25, 2002	:	**ATTN: BOX MISSING PARTS**

A SUSPENSION COMPRISING FIBRINOGEN, THROMBIN AND ALCOHOL, A METHOD FOR PREPARING SUCH A SUSPENSION, A METHOD FOR COATING A CARRIER WITH SUCH A SUSPENSION, A METHOD FOR DRYING A COATING OF A CARRIER, AND A COATED COLLAGEN SPONGE

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents,
Washington, DC 20231

Sir:

Pursuant to the provisions of 37 CFR 1.56, 1.97 and 1.98, Applicant requests consideration of [X] the references listed on attached form PTO-1449 and/or [] the additional information identified below in paragraph 3. A legible copy of each reference listed on the form PTO-1449 and each U.S. patent application listed below is enclosed, except a copy is not provided for each reference previously cited by or submitted to the Patent Office in prior parent application Serial No. .

1a. [X] This Information Disclosure Statement is submitted:

within three months of the filing date (or of entry into the National Stage) of the above-entitled application, **or**

before the mailing of a first Office Action on the merits or the mailing of a first Office Action after the filing of an RCE,

and thus no certification and/or fee is required.

1b. [] This Information Disclosure Statement is submitted

after the events of above paragraph 1a and prior to the mailing date of a final Office Action or a Notice of Allowance or an action which otherwise closes prosecution in the application, and thus:

(1) [] the certification of paragraph 2 below is provided, **or**

(2) [] the fee of $180.00 specified in 37 CFR 1.17(p) is enclosed.

1c. [] This Information Disclosure Statement is submitted:

after the mailing date of a final Office Action or Notice of Allowance or action which otherwise closes prosecution in the application, and prior to payment of the issue fee, and thus:

the certification of paragraph 2 below is provided, and

the fee of $180.00 specified in 37 CFR 1.17(p) is enclosed.

2. It is hereby certified

a. [] that each item of information contained in this Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the Statement, or

b. [] that no item of information contained in the Information Disclosure Statement was cited in a communication from a foreign patent office in a counterpart foreign application and, to the knowledge of the person signing the certification after making reasonable inquiry, was known to any individual designated in §1.56(c) more than three months prior to the filing of the Statement.

3. [] Consideration of the following list of additional information (including any copending or abandoned U.S. application, prior uses and/or sales, etc.) is requested.

4. For each non-English language reference listed on the attached form PTO-1449, reference is made to:

a. [X] a full or partial English language translation submitted herewith,

b. [] a foreign patent office search report (in the English language) submitted herewith,

c. [X] the concise explanation contained in the specification of the present application at page 3,

d. [X] the concise explanation set forth in the attached English language abstract,

e. [X] the concise explanation set forth below or on a separate sheet attached to the reference: Reference EP 0059265 corresponds to U.S. 4,453,939

5. [] A foreign patent office search report citing one or more of the references is enclosed.

Respectfully submitted,

Alfred SCHAUFLER

By _____

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April 22, 2002

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

Sheet 1 of 2

FORM PTO 1449 (*modified*)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

LIST OF REFERENCES CITED BY APPLICANT(S)
(*Use several sheets if necessary*)

Date Submitted to PTO: April 22, 2002

ATTY DOCKET NO.	SERIAL NO.
2002-0094A	10/054,889

APPLICANT
Alfred SCHAUFLER

FILING DATE	GROUP
January 25, 2002	1762

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	4,453,939	6/1984	Zimmerman et al.			
	AB	4,626,286	12/1986	Lubbs			
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	AD	5,618,551	4/1997	Tardy et al.			
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	AI						

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	AK	0578823 A1	1/1994	EP				
	AL	0059265	9/1982	EP				
	AM	0664132 A1	7/1995	EP			abstract	
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	AO	Chemical Abstracts, Columbus, Ohio, U.S., Vol. 98, June 13, 1983, No. 24.
	AP	"Mechanisms in blood coagulation, fibrinolysis and the complement system", Cambridge University press 1991; Halkier, Torben: Chapter 5: Activation of prothrombin, pgs.54-70.
	AQ	"Mechanisms in blood coagulation, fibrinolysis and the complement system", Cambridge University press 1991; Halkier, Torben: Chapter 7: Formation and stabillisation of fibrin, pgs. 80-103.

EXAMINER	DATE CONSIDERED

*EXAMINER: *Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.*

Sheet 2 of 2

FORM PTO 1449 (*modified*)

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FILING DATE	**GROUP**
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U.S. PATENT DOCUMENTS

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	AA						
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
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	AK	94/21739	9/1994	WO				
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	AM	97/37694	10/1997	WO				
	AN	99/13902	3/1999	WO				
	AO	1292326	10/1972	GB				

OTHER DOCUMENT(S) (*Including Author, Title, Date, Pertinent Pages, Etc.*)

	AP	"Mechanisms in blood coagulation, fibrinolysis and the complement system", Cambridge University press 1991; Halkier, Torben, Chapter 24: Haemostasis, pgs. 269-282.
	AQ	

EXAMINER	DATE CONSIDERED

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